*Journal of General Virology* (2004), **85**, 3195–3203 DOI 10.1099/vir.0.80159-0

# Bovine enterovirus 2: complete genomic sequence and molecular modelling of a reference strain and a wild-type isolate from endemically infected US cattle

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Bovine enteroviruses are members of the family *Picornaviridae*, genus *Enterovirus*. Whilst little is known about their pathogenic potential, they are apparently endemic in some cattle and cattle environments. Only one of the two current serotypes has been sequenced completely. In this report, the entire genome sequences of bovine enterovirus 2 (BEV-2) strain PS87 and a recent isolate from an endemically infected herd in Maryland, USA (Wye3A) are presented. The recent isolate clearly segregated phylogenetically with sequences representing the BEV-2 serotype, as did other isolates from the endemic herd. The Wye3A isolate shared 82 % nucleotide sequence identity with the PS87 strain and 68 % identity with a BEV-1 strain (VG5-27). Comparison of BEV-2 and BEV-1 deduced protein sequences revealed 72–73 % identity and showed that most differences were single amino acid changes or single deletions, with the exception of the VP1 protein, where both BEV-2 sequences were 7 aa shorter than that of BEV-1. Homology modelling of the capsid proteins of BEV-2 against protein database entries for picornaviruses indicated six significant differences among bovine enteroviruses and other members of the family *Picornaviridae*. Five of these were on the 'rim' of the proposed enterovirus receptor-binding site or 'canyon' (VP1) and one was near the base of the canyon (VP3). Two of these regions varied enough to distinguish BEV-2 from BEV-1 strains. This is the first report and analysis of full-length sequences for BEV-2. Continued analysis of these wild-type strains should yield useful information for genotyping enteroviruses and modelling enterovirus capsid structure.

Received 1 April 2004
Accepted 26 July 2004

## INTRODUCTION

The bovine enteroviruses (BEVs) are members of the family *Picornaviridae* that fall within the genus *Enterovirus*. Other viruses in this genus include porcine and simian enteroviruses, the human echoviruses, coxsackieviruses, the human enteroviruses and polioviruses, which are the most widely studied viruses of the genus (Pallansch & Roos, 2001). Originally classified into several serotypes, only two BEV serotypes are now recognized, based on updated assays and sequence analysis (Knowles & Barnett, 1985; McNally *et al.*, 1994; Zell & Stelzner, 1997). They share several features with other enteroviruses; however, they do not appear to cause major disease in cattle, although little is actually known regarding their pathogenesis within the enteric tract. They have been shown to be widely detectable in a farm environment and have thus been proposed as markers for fecal contamination from cattle (Ley *et al.*, 2002). It is generally assumed that BEVs are avirulent and, if so, it is possible that they would make useful vectors for delivery of small molecules to the gut. Additionally, BEVs have been shown to have oncolytic properties and may be useful as therapeutic agents (Shingu *et al.*, 1991; Smyth *et al.*, 2002). For these various reasons, our interest was sparked in learning more about the molecular anatomy of type 2 strains of BEV.

BEVs may be stable environmental contaminants on some cattle farms and, as such, might also serve as a useful surrogate for evaluating *Foot-and-mouth disease virus* (FMDV) farm contamination. FMDV belongs to the genus *Aphthovirus* (family *Picornaviridae*) and cannot be

The GenBank/EMBL/DDBJ accession numbers for the full-length BEV sequences reported in this study are AY508696 for BEV-2 PS87 and AY508697 for BEV-2 Wye3A.

studied on the US mainland. As such, on-farm studies to evaluate rapid detection approaches, efficacy of disinfectants or deactivators or extraction/detection approaches for FMDV cannot be carried out directly. Utilizing BEV as a surrogate might allow optimization of extraction and/or detection approaches for bovine picornavirus contamination in the farm environment (Lund *et al.*, 1996; Monteith *et al.*, 1986; Yilmaz & Kaleta, 2003).

Study of BEV has also provided significant data of use to picornavirus virologists (Kaminaka *et al.*, 1999; Rohll *et al.*, 1995; Zell & Stelzner, 1997; Zell *et al.*, 1999). The crystal structure of the BEV virion was deduced from crystals of purified BEV-1 strain VG5-27 (Smyth & Martin, 2001, 2002; Smyth *et al.*, 1993) and comparisons with the capsid structure of poliovirus indicate a variety of similarities and some distinct differences in the antigenic and receptor-binding regions of the virus. We decided to compare genomic sequences of virus isolates from a local farm that is known to be endemically infected with BEV to the ATCC isolate BEV-2 PS87, originally from Pennsylvania, USA (Dunne *et al.*, 1974), and to evaluate the differences between these US isolates.

Further, as no full-length genome sequence for BEV-2 was available, we completed the entire genome sequence of two strains to enhance the database of sequences of members of the family *Picornaviridae*. Having obtained these new sequences, we were able to generate and compare structural models by using selected picornavirus templates from the Protein Database (PDB) structure coordinates database.

## METHODS

**Viruses.** Wild-type BEV isolates were selected from fecal samples of 2–4-month-old Angus calves at the Wye Research Farm (University of Maryland, MD, USA). Animal and environmental samples from the farm were identified as described previously (Ley *et al.*, 2002). Briefly, 1 g fecal material was diluted in 10 ml PBS, centrifuged at 1800 ***g*** and filtered through a 0·45 μm filter. Madin–Darby bovine kidney (MDBK) cells in Eagle's minimal essential medium supplemented with horse serum (Gibco) were infected with 100 μl filtered supernatant. The reference strain BEV-2 PS87 (ATCC catalogue no. VR-774) was used as a positive control. Supernatant from MDBK cells with cytopathic effect (CPE) after 3 days incubation were collected and frozen at −70 °C. Both BEV-2 PS87 and Wye3A strains were plaque-purified and subsequently passaged (P2); this stock was used for sequence analysis.

**RNA amplification and sequencing.** RNA from infected MDBK cell supernatant was extracted with TRIZOL LS reagent (Invitrogen) according to the manufacturer's instructions, with the exception of the addition of 1 μl glycogen (20 mg $ml^{-1}$) at the chloroform-extraction phase. RNA pellets were resuspended in 20 μl DEPC-treated water. The presence of BEV was confirmed by RT-PCR using Titan One-Step RT-PCR (Roche) according to the manufacturer's protocol and primers corresponding to positions 1–26 and 979–1000 of a previously published partial sequence of BEV-2 PS87 (McNally *et al.*, 1994).

Real-time RT-PCR primers were designed to previously published partial sequences of BEV-2 (McNally *et al.*, 1994) and BEV-2 261 (GenBank accession no. AJ250672), and to conserved regions of BEV-1 [VG5-27 (Earle *et al.*, 1988), SL305 and K2577 (McCarthy *et al.*, 1999)], BEV-2 Bot 209 (GenBank accession no. AJ250673) and BEV-1 BEV1 (GenBank accession no. AJ250671). The forward primer sequence corresponds to nt 271–288, the reverse primer to nt 554–569 and the 6FAM- and TAMRA-labelled probe to nt 373–394. Real-time RT-PCR was performed by using Titan One-Step RT-PCR (Roche) according to the manufacturer's protocol with 1 μM primer and 0·2 μM probe. Cycling conditions run on a Stratagene Mx4000 thermal cycler were: 30 min at 48 °C preceded 40 cycles of 95 °C for 30 s, 50 °C for 30 s and 60 °C for 45 s.

**Nucleotide sequence analysis.** Extracted RNA samples were amplified by using RT-PCR as described above, using primers that were initially designed to correspond to conserved regions of published BEV-1 and BEV-2 sequences and subsequently to derived sequences. Primer sequences are available from the authors upon request. Amplicons from positive reactions were purified with a High Pure PCR purification kit (Roche) and cycle-sequenced by using a Big Dye Terminator cycle sequencing kit version 3.1 and an ABI 3100 genetic analyser (both from Applied Biosystems). Three or more amplicons were used to arrive at an overlapping consensus sequence, analysed with Sequencher 4.41 software (GeneCodes). A RACE kit (Invitrogen) and a Topo TA cloning kit for sequencing (Invitrogen) were used to complete and verify the extreme 3′ and 5′ ends of the genomes.

**Phylogenetic analysis of BEV-2 and other picornaviruses.** Nucleotide sequences were aligned and compared by using the program CLUSTAL W. The program Mfold 3.1 (Zuker, 2003) was used to generate and compare predicted secondary structures of the 5′ untranslated region (UTR) RNA sequences of the BEV strains. For analysis of full-length polyprotein sequences from picornaviruses, the program PAUP version 4 (Sinauer Associates, Sunderland, MA, USA) was used to perform heuristic searches, neighbour-joining and bootstrap analyses. Phylogenetic trees were generated in Lasergene (MEGALIGN), PAUP or by the TreeView program (Page, 1996).

**Modelling of capsid proteins.** For the purpose of VP1–4 structure modelling, all 79 protein structures that were related to bovine enterovirus VG5-27 and deposited in PDB were considered as potential homology templates. All of these templates were examined to select the final set of structures that would be useful for modelling. Detailed structural analysis of all templates allowed definition of regions of structural conservation and regions of structural diversity between templates. The resolution of all considered templates varied from 2·15 to 3·55 Å. In addition to X-ray-solved structures, three cryo-electron microscopy (CRYO-EM) structures were also used to verify the conformation of components VP1–4 within the modelled protomer complex.

For modelling protein capsids VP1–4, the following templates were used: 1bev, BEV-1 VG5-27, X-ray 3·00 Å; 1hxs, Human poliovirus 1 (HPV-1) Mahoney, X-ray 2·20 Å; 1aym, human rhinovirus 16 (HRV-16), X-ray 2·15 Å; 1ar7, HPV-1 Mahoney (double mutant), X-ray 2·90 Å.

In addition to the structural templates above, the following protein structures were used for model templates structure comparison to detect regions of structural deviation: 1d4m, CV-A9, X-ray 2·90 Å; 1al2, HPV-1 Mahoney, X-ray 2·90 Å; 1ev1, EV-1, X-ray 3·55 Å; 2plv, HPV-1 Mahoney, X-ray 2·88 Å; 1pvc, HPV-3 Sabin, X-ray 2·40 Å; 1oop, PEV-9 (swine vesicular disease virus), X-ray 3·00 Å; 1cov, CV-B3 coat protein, X-ray 3·50 Å; 1dgi, HPV-1, CRYO-EM (fitting of X-ray 2·88 Å PDB-2plv structures); 1jew, CV-B3 (M strain), CRYO-EM (fitting of X-ray 3·50 Å PDB-1cov structures); 1m11, EV-7, CRYO-EM (fitting of X-ray 3·55 Å PDB-1ev1 structures).

Capsid proteins from BEV-2 strains PS87 and Wye3A were modelled by using the AS2TS homology modelling system (Zemla, 2003), by which protein sequences are compared against all PDB entries and those with the highest homology are evaluated as suitable templates for modelling. PDB entry 1bev (BEV-1 VG5-27) had the highest sequence identity for the BEV-2 capsid proteins, although template 1hxs_4 of poliovirus (Mahoney strain) was substituted for 1bev_4, as the former was more complete and had been solved at higher resolution (see below). In regions where residues had been omitted from the X-ray crystallography data, additional analyses using Local–Global Alignment (LGA) software (Zemla, 2003) and secondary-structure prediction using PSIPRED (Jones, 1999) were done in order to attempt completion of the templates. Backbone models consisting of main-chain atoms for the capsid proteins of BEV strains PS87, Wye3A and K2577 were then constructed by using the modified templates. Loop regions were constructed by using the LGA modeller program. The side-chain atoms for residues that were identical to those of the templates were incorporated into the models automatically and the remaining side-chain atoms were calculated by using the program SCWRL (Bower *et al.*, 1997).

## RESULTS

### Transient appearance of BEV in calves from an endemically infected herd

Real-time RT-PCR (Table 1) and cell-culture analysis of fecal extracts of 3- and 7-month-old calves clearly indicate widespread infection of Wye farm cattle with BEV, supporting the results of previous surveys (Ley *et al.*, 2002). The data also suggest that the presence of the virus is transient in animals. At 3 months of age (in May), 50 % of calves yielded a positive real-time RT-PCR profile. Cell-culture assays of the same samples indicated that at least 50 % of the samples also had positive, typical CPE (data not shown). At 7 months of age (in September), none of the calves sampled was positive by PCR. At both sampling times, however, BEV was detected in the farm water-troughs by RT-PCR, indicating either that the virus may persist in the environment for longer periods or that some animals continue to shed virus (data not shown). Cell-culture analysis was sometimes difficult to interpret because of some non-specific cell death that was caused by the fecal extracts, but when samples that were positive for both real-time PCR and CPE were passaged, several isolates that were determined to be BEV-2 by sequence analysis were obtained.

**Table 1.** BEV detection by RT-PCR from Angus calves

| Collection date | No. calves sampled* | No. extracts with positive RT-PCR signal | Range of genome copies (ml fecal extract)$^{-1}$ |
|---|---|---|---|
| April 2003 | 29 | 15 | 3–150 |
| September 2003 | 34 | 0 | NA |

*Six calves were sampled both in April and September. Three of these were positive in April and negative in September.

### Sequence and characteristics of two BEV-2 genomes

A summary of the sequence information determined for the two fully sequenced strains of BEV-2 is shown in Table 2. Although there were observable differences in the nucleotide sequences of the UTRs (particularly so with the 3′ UTRs), comparison of the predicted secondary structures of the 5′ and 3′ UTR sequences from BEV-1 and BEV-2 using the program Mfold (Zuker, 2003) revealed that the predicted RNA-folding structures were similar and there was no indication of variation from the secondary structure that was predicted previously for BEV-1 (Zell *et al.*, 1999). Both BEV-2 polyprotein ORFs were 27 nt shorter than those for BEV-1. The difference was found primarily in the VP1 coding region, where BEV-2 had 21 deleted nucleotides compared to BEV-1. Alignments using CLUSTAL W indicated that these deletions clustered near the N-terminal coding region of VP1 (Fig. 1). The deletion proposed in Fig. 1 is based upon a comparison of codons in the strains. Other deletions could be predicted by altering the CLUSTAL W parameters.

Additional differences noted in the sequences of BEV-1 and BEV-2 were the presence of glutamine-glycine at the proposed BEV-2 cleavage site for VP3/VP1, where BEV-1 has glutamine-asparagine, and at the 2B/2C cleavage site, where BEV-2 has glutamine-alanine, rather than glutamine-serine for BEV-1. All other predicted polyprotein cleavage sites for $3C^{pro}$ were of the more common glutamine-glycine type that is found for most picornaviruses.

Table 3 illustrates sequence similarities among the completely sequenced BEV strains and other selected enteroviruses. The

**Table 2.** Summary of genomic sequences of BEV isolates

| Isolate | Feature | Length (nt) |
|---|---|---|
| BEV-1 VG5-27 | ORF | 6525 |
| | 5′ UTR | 818 |
| | 3′ UTR | 71 |
| | Poly A | ? |
| | Total | 7414 |
| BEV-2 PS87 | ORF | 6498 |
| | 5′ UTR | 819 |
| | 3′ UTR | 73* |
| | Poly A† | ⩾36 |
| | Total | 7426 |
| BEV-2 Wye3A | ORF | 6498 |
| | 5′ UTR | 820 |
| | 3′ UTR | 74* |
| | Poly A† | ⩾58 |
| | Total | 7450 |

*Includes 3′ stop codon.

†Maximum number of adenosine residues amplified with 3′ RACE. The actual length of the poly A tail was not determined.

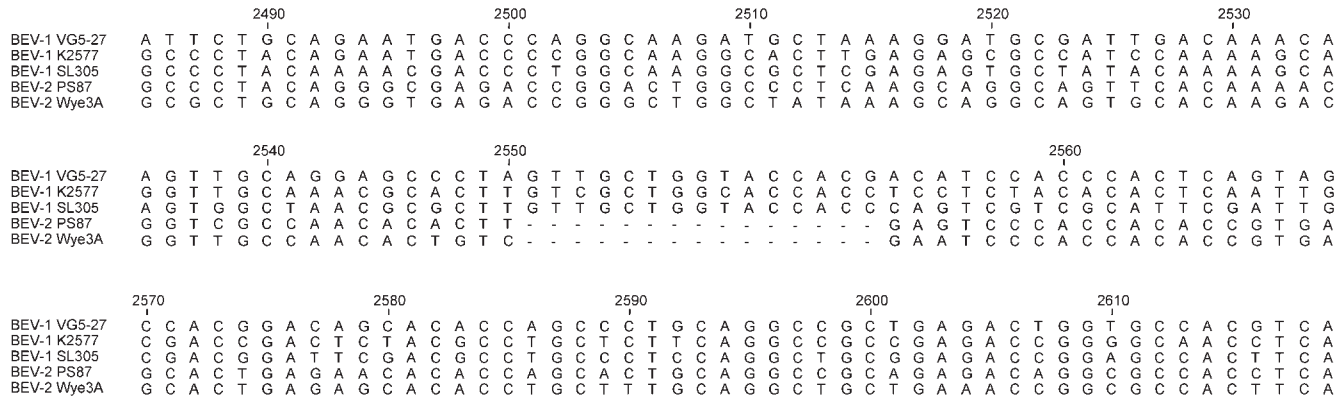


**Fig. 1.** Alignment of the variable N-terminal region of VP1 gene sequences from BEV-1 and -2 strains. The N-terminal amino acid of VP1 begins at nt 2494. Numbering is based on the PS87 sequence.

**Table 3.** Nucleotide and predicted amino acid sequence percentage identities among selected enteroviruses

For the 5′ UTR and 3′ UTR comparisons, values above the diagonal are 5′ UTR nucleotide sequence identities and values below the diagonal are 3′ UTR nucleotide sequence identities. For the other comparisons, values above the diagonal are nucleotide sequence identities and values below the diagonal are amino acid sequence identities.

| Sequence identity (%) | Polio1 | BEV-1 VG5-27 | BEV-1 K2577 | BEV-2 PS87 | BEV-2 Wye3A | PEV-9 |
|---|---|---|---|---|---|---|
| **UTRs** | | | | | | |
| Polio1 | | 49·7 | 44·3 | 46·5 | 46·5 | 58·4 |
| BEV-1 VG5-27 | 13·0 | | 78·2 | 74·3 | 78·7 | 45·2 |
| BEV-1 K2577 | 21·7 | 95·7 | | 78·4 | 74·1 | 41·9 |
| BEV-2 -PS87 | 21·7 | 76·8 | 76·8 | | 87·8 | 42·9 |
| BEV-2 Wye3A | 15·9 | 81·2 | 81·2 | 97·3 | | 45·4 |
| PEV-9 | 11·6 | 68·1 | 71·0 | 57·5 | 68·5 | |
| **P1** | | | | | | |
| Polio1 | | 33·7 | 32·7 | 50·6 | 50·8 | 50·4 |
| BEV-1 VG5-27 | 44·9 | | 73·2 | 63·3 | 64·4 | 62·8 |
| BEV-1 K2577 | 44·5 | 86·5 | | 63·2 | 64·3 | 61·8 |
| BEV-2 PS87 | 46·4 | 65·9 | 66·5 | | 82·0 | 61·1 |
| BEV-2 Wye3A | 45·7 | 66·6 | 66·5 | 95·4 | | 62·2 |
| PEV-9 | 43·2 | 60·8 | 62·0 | 60·5 | 60·8 | |
| **P2** | | | | | | |
| Polio1 | | 59·1 | 59·7 | 60·4 | 59·2 | 56·6 |
| BEV-1 VG5-27 | 55·8 | | 80·5 | 68·8 | 68·7 | 61·7 |
| BEV-1 K2577 | 56·0 | 96·5 | | 69·3 | 68·6 | 61·7 |
| BEV-2 PS87 | 56·9 | 75·6 | 76·1 | | 81·0 | 60·4 |
| BEV-2 Wye3A | 56·9 | 75·4 | 76·0 | 96·7 | | 63·5 |
| PEV-9 | 53·7 | 62·3 | 62·8 | 61·6 | 61·2 | |
| **P3** | | | | | | |
| Polio1 | | 60·7 | 61·9 | 64·2 | 63·7 | 63·6 |
| BEV-1 VG5-27 | 62·7 | | 80·9 | 69·4 | 69·5 | 65·0 |
| BEV-1 K2577 | 62·9 | 97·2 | | 69·5 | 69·7 | 64·1 |
| BEV-2 PS87 | 64·1 | 77·9 | 79·1 | | 84·0 | 65·8 |
| BEV-2 Wye3A | 64·1 | 78·0 | 79·0 | 97·6 | | 65·8 |
| PEV-9 | 65·3 | 69·7 | 69·9 | 70·5 | 70·5 | |

genomes were divided into 5′ and 3′ UTRs and P1, P2 and P3 subgenomic regions. Comparing the P1 region, which encodes VP4, VP2, VP3 and VP1, the two BEV-1 isolates shared 73·2 % nucleotide and 86·5 % amino acid identity and the two sequenced BEV-2 isolates shared 82·0 % nucleotide and 95·4 % amino acid identity. The BEV-2 strains shared 63·2–64·4 % nucleotide identity and 65·9–66·6 % amino acid identity with the BEV-1 strains. Whilst BEV-1 strains shared only 32–33 % nucleotide identity with HPV-1 (Polio1), BEV-2 strains shared approximately 50 % nucleotide sequence identity with HPV-1; a similar nucleotide sequence identity is shared between PEV-9 and HPV-1. Although the nucleotide sequences varied, comparisons of the predicted amino acid sequences found that all of the enteroviruses compared in this analysis were equally similar, sharing approximately 45 % identity. CLUSTAL W alignments of nucleotide and amino acid sequences of the P2 and P3 regions [which encode the non-structural proteins, VPg (a structural protein), protease and polymerase] yielded similar results. The two BEV-2 isolates clearly grouped together and were separate from the BEV-1 strains, and the four BEV strains grouped together, separate from PEV-9 and HPV-1 (Table 3).

## Phylogenetic relationship of Wye farm isolates with other BEV-2 strains and enteroviruses

Phylogenetic relationships of the sequenced strains are consistent with previously determined BEV serotype relationships, in that there are two clear lineages. The 5′ UTR sequences of the Wye farm isolates segregated with all the partial sequences from available BEV-2 database entries, rather than with any of the BEV-1 strains (Fig. 2a). This is similar to the data that were reported from the same farm 2 years earlier (Ley *et al.*, 2002). Similarly, comparison of full-length nucleotide sequences also demonstrated clear separation of BEV-1 from BEV-2 (Fig. 2b). Comparison of predicted amino acid sequences representing the polyprotein from several enteroviruses in the NCBI database by using PAUP (Fig. 3) and rooted by using porcine teschovirus 1 (PTV-1) demonstrated that the BEV-2 strains co-segregated in a clade that included BEV-1, porcine

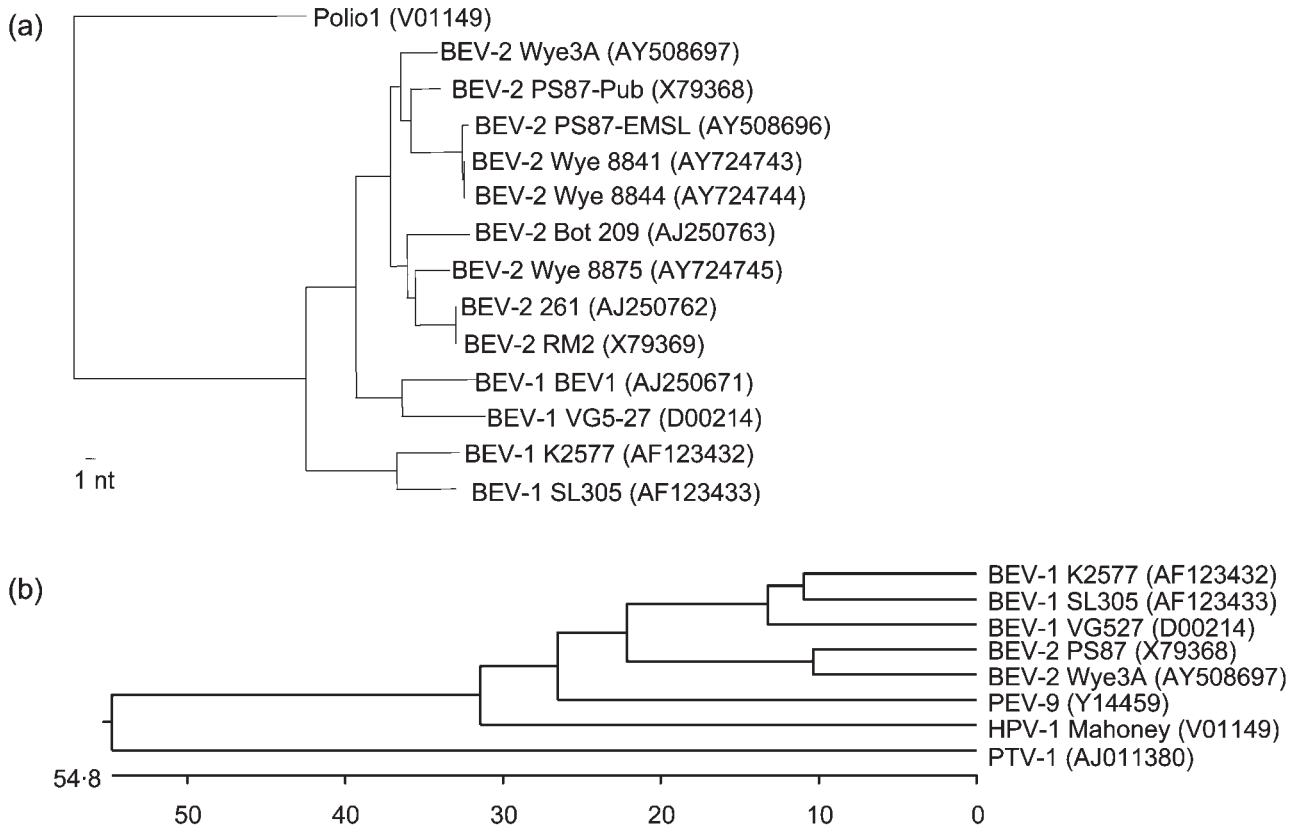


**Fig. 2.** Phylogenetic relationships among sequenced strains of BEV-1 and BEV-2. Trees were derived from CLUSTAL W alignments by using the unweighted pair group method and the tree-drawing program in DNASTAR. (a) 5′ UTR sequences that were common to all NCBI database and sequenced strains (227 nt). (b) Full-length sequences. Strain designations were taken from GenBank (see Methods) except for BEV-2 Wye3A and PS87-EMSL, the strains described and sequenced in this report. PS87-Pub is the BEV-2 PS87 sequence obtained from GenBank.

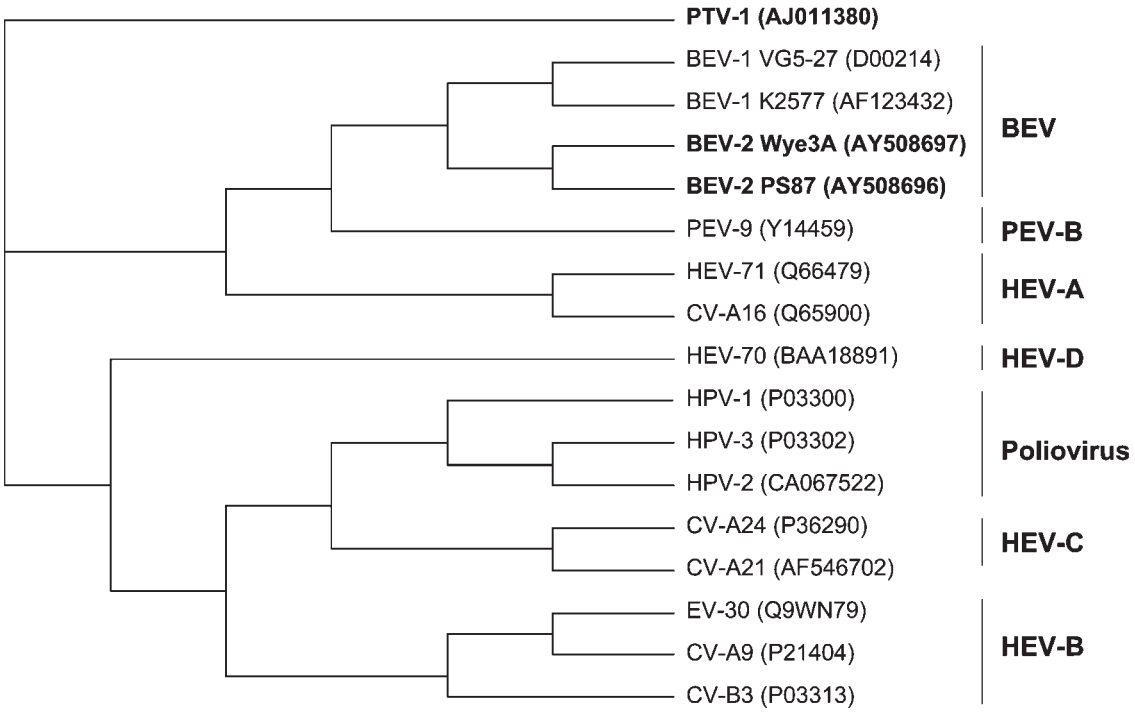


**Fig. 3.** Phylogenetic relationships of the polyprotein encoded by selected strains of enteroviruses. PAUP-generated tree following neighbour-joining analysis, heuristic search and bootstrap analysis for the full polyprotein.

enterovirus and the *Human enterovirus A* (HEV-A) strains. This clade was distinct from the clade containing poliovirus, HEV-D, HEV-C and HEV-B strains, consistent with prior analyses (Stanway *et al.*, 2002).

## Capsid protein modelling of BEV-2 isolates

Capsid proteins VP1, VP2, VP3 and VP4 of BEV-2 strains Wye3A and PS87 were modelled by using templates that were derived from the PDB crystal structures of BEV-1 (VG5-27) entries 1bev_1, 1bev_2, 1bev_3 and 1hxs_4 of PV1-Mahoney, respectively. Additional templates were identified as needed to complete regions of ambiguity or regions that were missing from primary templates. The modified templates then were used to construct homology models for each capsid protein of strains PS87 and Wye3A. The modified 1bev template will be deposited in PDB and is available from the authors upon request. Details on template modification follow.

**Template VP1.** As 13 N-terminal residues of 1bev chain '1' (VP1) were not provided in the PDB entry and temperature factors for atoms of residues 14–17 were very high (above 60), additional analyses were required to model this region. In summary, we examined several PDB templates and ultimately determined that residues 4–17 were best modelled as an α-helix by using PDB structure 1aym chain '1' as a template.

**Template VP2.** Residues 1–4 of 1bev chain '2' (VP2) were not provided in the PDB entry. We were unable to model these residues, due to lack of a PDB homologue for which coordinates were provided for the corresponding residues. The high temperature factors (low confidence) for residues 5–11, however, prompted us to adjust the structural assignment of these residues. We identified 1ar7 chain '2' as the best template for modelling this region and adjusted 1bev chain '2' coordinates accordingly. This new conformation was also found to avoid clashes with the reconstructed C-terminal disordered region of 1bev chain '4'.

**Template VP3.** There were no missing residues in the PDB entry for 1bev chain '3' (VP3). Therefore, the VP3 proteins for the three strains were modelled completely with high confidence by homology to 1bev chain '3'.

**Template VP4.** It was determined that 1bev chain '4' (VP4) was a poor template for modelling VP4, due to the lack of coordinate data for 28 of 68 residues and due to very high temperature factors for most of the residues in the PDB entry. Of 25 structures of VP4 homologues that were selected from PDB, we determined that entry 1hxs of poliovirus (Mahoney strain), with 57 % sequence identity to 1bev chain '4' and solved with 2·2 Å resolution, was the most complete and would provide the best template for modelling most regions of VP4 proteins of the three strains.

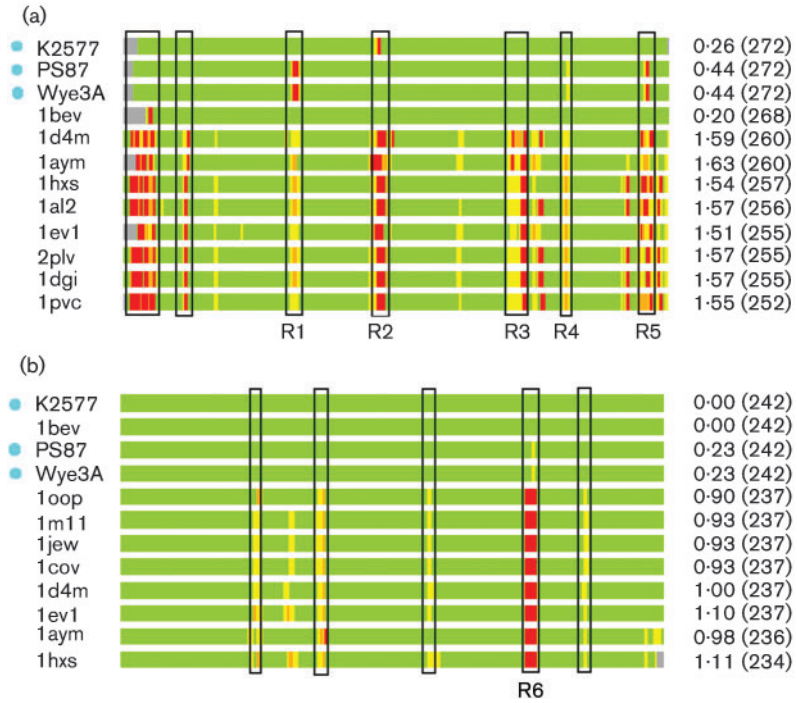


**Fig. 4.** Structure comparison of template VP1 (a) and template VP3 (b) with corresponding VP1 and VP3 capsid proteins from BEV variants and other picornaviruses from PDB (our theoretical models are marked with cyan bullets). Coloured bars show the results of pairwise structure comparisons and calculated root mean square deviation values are on the right side of each bar [the calculated number of structurally aligned residues is provided in parentheses and only residues that correspond to the frame of reference (our VP1 and VP3 templates) are presented in bars]. Residues superimposed at the distance deviation below 2·00 Å are in green, below 4·00 Å in yellow, below 6·00 Å in orange and residues superimposed above 6·00 Å or not aligned are in red. Bars are arranged in order by LGA_S score from highest to lowest (data not shown). Regions showing considerable structural deviation among BEVs or between BEVs and other picornaviruses are boxed. Labelled regions (R1–R6) correspond to surface-exposed residues on the capsid (see Fig. 5). (a) From top down, bars represent BEV-1 strain K2577, BEV-2 strain PS87, BEV-2 strain Wye3A and PDB templates 1bev, 1d4m, 1aym, 1hxs, 1al2, 1ev1, 2plv, 1dgi and 1pvc. (b) From top down, bars represent BEV-1 strain K2577, PDB template 1bev, BEV-2 strain PS87, BEV-2 strain Wye3A and PDB templates 1oop, 1m11, 1jew, 1cov, 1d4m, 1ev1, 1aym and 1hxs.

### Structural deviations among the capsid proteins of BEV-2 isolates and other picornaviruses

When compared with other picornaviruses, capsid proteins VP1 and VP3 of BEV-1 and -2 deviated considerably in six regions (R1–R6; see Fig. 4) (residue numbering corresponds to the 1bev structure from PDB). N-terminal regions were not considered to represent significant structural deviations, as they are expected to be flexible and difficult to solve with high confidence. These six regions lie at or within the so-called canyon, which is believed to be involved in capsid protein–host receptor binding (Smyth & Martin, 2001; Rossmann, 2002) and to confer antigenic variation. Four of these regions (R1: 86–92, R2: 127–138, R4: 226–230 and R5: 266–273) have various insertions or deletions with respect to the amino acid sequence of entry 1bev chain '1' and, for VP3, there was an insertion in a loop region of the BEV-2 strains relative to K2577 (R6: 180–187). Region R3 (198–208) exhibits structural variation among the considered PDB templates and overlaps with the region of sequence variation between the two BEV strains. Each of these regions is located on the surface of the 1bev protomer (Fig. 5), either on a 'rim' of the canyon or at its base. The structural models of the capsid proteins of PS87, Wye3A and K2577 farm isolates are nearly identical, as they were built by using the same modified 1bev template. However, there are clear variations in structure between BEV-1 and BEV-2 strains in insertion/deletion regions and structural variation in regions identified by sequence variation (e.g. region R3: 198–208).

## DISCUSSION

The relative lack of information on BEV pathogenesis in bovine populations has resulted in little study on the molecular biology of these viruses. However, the BEV-1 capsid has been crystallized and the structure solved to 3·5 Å resolution, allowing comparison with other picornavirus structures, and has yielded useful information (Smyth & Martin, 2002; Smyth *et al.*, 1995). In this study, determination of the complete sequence of the ATCC BEV-2 strain PS87 and a recent wild-type isolate has provided a

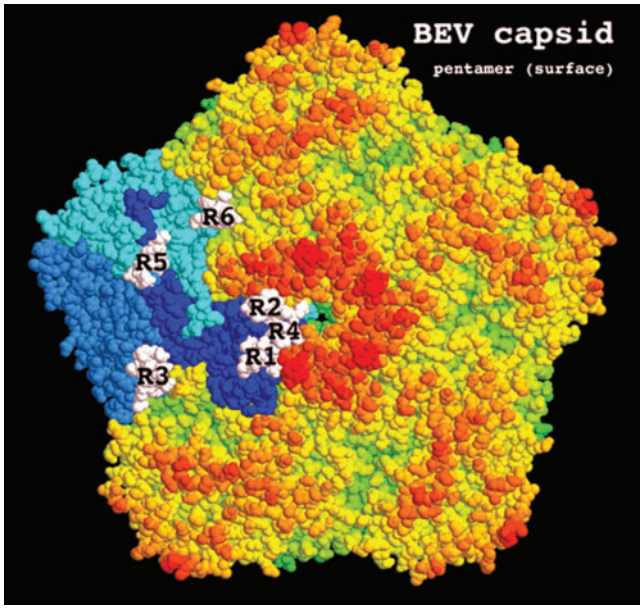


**Fig. 5.** The surface of the BEV capsid (pentamer subunit) constructed by using the modified 1bev template. Topology is indicated by red–green contrast, with red representing the most exposed surface atoms and green representing depressions in the capsid surface. A single protomer is depicted in shades of blue: VP1 is in dark blue, VP2 in medium blue and VP3 in light blue. Protomer component protein VP4 is not visible because it lies on the inner surface of the capsid. Regions of structural deviation between BEV strains and other picornaviruses (R1–R6) and among BEV strains (R1, R2, R4, R5, R6) are shown in white. Regions R1–R5 are located on the rims of the observed 'canyon', with R6 lying in its base.

clearer picture of the BEVs. The nucleotide and predicted amino acid sequence determined for the PS87 strain that was obtained from ATCC was somewhat different from the previously published partial sequence (McNally *et al.*, 1994). Our sequence was derived directly from several overlapping RT-PCR amplicons that represented the majority population of a low-passage, plaque-purified stock (P3 in our laboratory), whereas the published sequence was apparently derived from cloned PCR products. It is likely that the number of products and method of nucleotide sequencing and/or the passage histories of the respective virus samples analysed may be responsible for the observed differences.

Phylogenetically, all of the viruses that were isolated from the Wye farm were clearly shown to be BEV-2 strains, as shown here and previously (Ley *et al.*, 2002). BEV-specific RT-PCR suggested that the virus was present in the spring in 2–4-month-old calves and in the environment, but the infection had probably been cleared by summer. We are interested in these wild-type isolates in terms of prevalence, pathogenesis and their potential as vectors for expressing small molecules. Studies are under way to evaluate infectious clones of various BEV-2 strains, both as vectors and as tools to study potential pathogenesis.

Analysis of the full-length and subgenomic region nucleotide and predicted amino acid sequences of PS87 and Wye3A demonstrated that they belong to the same genotypic group. The BEV genotypes segregated as the virus isolates (of known serotype) have been shown to segregate by serotype. Although we have only looked at a limited number of BEV isolates and the serotype of the Wye3A isolate has not been confirmed, the data suggest that there is a clear difference in nucleotide and amino acid sequence between BEV-1 and BEV-2 strains and that this difference probably reflects the antigenic differences that have been observed between serotypes 1 and 2.

The difference in size between the VP1 proteins of BEV-1 and BEV-2 is noteworthy. The sequence comparisons in Fig. 1 suggest that substantial nucleotide deletions have occurred near the N-terminal coding sequences of BEV-2 between positions 2550 and 2610. By using CLUSTAL W on default settings, a 5 aa deletion in BEV-2 relative to BEV-1 was detected near the N terminus (data not shown). However, the sequence-based alignment is unreliable in this region and is partly dependent on parameter settings. Therefore, a precise correspondence between the N-terminal residues of the BEV-2 and BEV-1 VP1 proteins could not be determined. Analyses using various sequence alignment search techniques (e.g. PSI-BLAST, CLUSTAL W) and homology modelling revealed structural ambiguity inasmuch as several PDB structures of VP1 consisted of α-helix in this region, whereas several others consisted of strand or disordered regions. Based on comparative analysis of PDB templates, we determined that the most reasonable solution was to model the four N-terminal residues of 1BEV_1 (NDPG) as strand and residues 5–17 as α-helix. However, because the BEV-2 proteins were 5 aa shorter than those of BEV-1, it was not possible to assign these residues with certainty to either the strand portion or the α-helix portion of the template. We chose to maintain continuity at the N terminus and align these residues with the α-helix portion of the modified 1BEV_1. Most data have suggested that this N-terminal region of VP1 would be internal and disordered, and may make up a protein layer that separates the external capsid from the RNA (Rossmann, 2002). It would therefore be interesting to determine whether these differences are significant to the structure of the two BEV serotypes.

Molecular modelling of the BEV-2 capsid structure and structural comparisons with picornaviruses support the generally accepted idea that the region of VP1 that connects the eight β-strands making up the wedge-shaped region of each capsid protein is part of the variable region, specifying the antigenically variable sites (Rossmann, 2002). Most regions of structural variation detected by our structure alignment analysis (Fig. 4) lie in this variable region around the canyon, and region R3 overlaps with an antigenic site 1 that was described previously (Smyth & Martin, 2001). The variations described (particularly in R1 and R5) are probably the differences that specify the

antigenic nature and separation of the two BEV serotypes. The significance of the difference in the structure of VP3 that appears near the bottom of the canyon is not known, but might have some influence on the receptor-binding capacity. Studies are sparse regarding the receptor specificity of BEV, but early experiments suggested that sialic acid might be a receptor (reviewed by Racaniello, 2001).

The phylogenetic and structural differences found between BEV-1 and BEV-2 in this study support a distinct separation of the two groups, but very little is known regarding these relationships with respect to serotypic relationships. It has been suggested that antigenic properties as classification criteria for picornaviruses will become unnecessary as sequence relationships become clearer (Stanway *et al.*, 2002). Our data would support this notion. As we know that BEVs continue to be found in US cattle and are shed into the environment, it is worthwhile to continue to collect and analyse them. As they may share a common ancestor with and form a distinct clade with some human enteroviruses (HEV-A, Fig. 3) and as there are virtually no serological or epidemiological data on the extent of BEV infection in US cattle, it is certainly worth continuing to be proactive in the characterization of these viruses and their potential pathogenesis.

## ACKNOWLEDGEMENTS

We are grateful to Mr Eddie Draper and the staff at the University of Maryland Wye Farm for their assistance in collecting samples. We thank Brent Becker for excellent technical assistance.

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